American Journal of Pathology, Vol. 166, No. 4, April 2005


*Immunopathology and Infectious Disease*

# Contribution of Transmembrane Tumor Necrosis Factor to Host Defense against *Mycobacterium bovis* Bacillus Calmette-Guerin and *Mycobacterium tuberculosis* Infections

Maria L. Olleros,* Reto Guler,* Dominique Vesin,* Roumen Parapanov,* Gilles Marchal,† Eduardo Martinez-Soria,* Nadia Corazza,‡ Jean-Claude Pache,* Christoph Mueller,‡ and Irene Garcia*

*From the Department of Pathology and Immunology,* University of Geneva, Geneva, Switzerland; the Department of Pathology,‡ University of Bern, Bern, Switzerland; and the Laboratoire du Bacillus Calmette-Guerin and Unité de Physiopathologie de l'Infection,† Institut Pasteur, Paris, France*

**To study the specific role of transmembrane tumor necrosis factor (TmTNF) in host defense mechanisms against bacillus Calmette-Guerin (BCG) and *Mycobacterium tuberculosis* infections, we compared the immune responses of TNF/lymphotoxin (LT)-$\alpha^{-/-}$ mice expressing a noncleavable transgenic TmTNF (TmTNF tg) to those of TNF/LT-$\alpha^{-/-}$ and wild-type mice. Susceptibility of TNF/LT-$\alpha^{-/-}$ mice to BCG infection was associated with impaired induction of systemic RANTES but not of monocyte chemoattractant protein 1 (MCP-1), the development of excessive local and systemic Th1-type immune responses, and a substantially reduced inducible nitric oxide synthase (iNOS) activity. Resistance of TmTNF tg mice to BCG infection was associated with efficient activation of iNOS in granulomas and with the regulated release of local and systemic chemokines and Th1-type cytokines. However, *M. tuberculosis* infection of TmTNF tg mice resulted in longer survival and enhanced resistance compared to TNF/LT-$\alpha^{-/-}$ mice but higher sensitivity than wild-type mice. TmTNF tg mice exhibited reduced pulmonary iNOS expression and showed an exacerbated cellular infiltration in the lungs despite a modest bacillary content. Our data thus indicate a role for TmTNF in host defense against mycobacteria by contributing to induction and regulation of Th1-type cytokine and chemokine expression leading to development of bactericidal granulomas expressing iNOS, which critically determines susceptibility versus resistance of the host to mycobacterial infections. *(Am J Pathol 2005, 166:1109–1120)***

Tuberculosis is re-emerging as one of the most important health problems. Approximately 2 million people die each year from this curable disease and there are at least 8 million new cases per year.[1] Human immunodeficiency virus and the emergence of multidrug-resistant strains are contributing to the increase of tuberculosis mortality. The only available vaccine against *Mycobacterium tuberculosis*, *Mycobacterium bovis* bacillus Calmette-Guerin, has marked variable effectiveness.[2]

Murine models of experimental tuberculosis have allowed the understanding of part of the mechanisms implicated in mycobacterial infections, as the induction of Th1 cell-mediated immune responses, characterized by the presence of interferon (IFN)-$\gamma$.[3,4] Mycobacteria are isolated in protective granulomas,[5] consisting of differentiated macrophages and T cells, and killed by bactericidal mechanisms such as inducible nitric oxide synthase (iNOS), the enzyme producing nitric oxide (NO). The iNOS plays a crucial role in host defense against mycobacterial infections,[6,7] and is synergistically activated by tumor necrosis factor (TNF) and IFN-$\gamma$.[8]

TNF is first synthesized as a 26-kd transmembrane (TmTNF) precursor and cleaved by membrane-bound metalloprotease-disintegrin TACE (TNF-$\alpha$-converting enzyme) generating a soluble 17-kd TNF molecule.[9,10] Most TNF activities were attributed to the secreted or soluble (sTNF) form, particularly those related to resistance to

Supported by the Swiss National Foundation for Scientific Research (grant 3200B0-105914 to I.G.), the Roche Foundation, the E. and L. Schmidheiny Foundation, and the Jules Thorn Foundation.

Accepted for publication January 10, 2005.

Address reprint requests to Dr. Irene Garcia, Department of Pathology and Immunology, Centre Medical Universitaire, 1 rue Michel-Servet, CH 1211 Geneva 4, Switzerland. E-mail: irene.garcia-gabay@medecine.unige.ch.

intracellular bacterial infections such as mycobacteria. Animal studies, including our previous works, have provided compelling evidence implicating sTNF as a key factor in host defense against mycobacterial infections. Impaired granuloma formation and bactericidal mechanisms, and alteration of mycobacterium-induced Th1-type immune responses were observed in animals unable to use TNF.[11–21] The differential contribution of TmTNF and sTNF in mycobacterial host defense has not been studied in detail. We previously showed that TmTNF, in the absence of sTNF and lymphotoxin (LT)-$\alpha$, induces an efficient cell-mediated immune response to *M. bovis* BCG infection.[20] This study was performed using TmTNF transgenic mice in which expression of the transgene is controlled by the TNF promoter and the 3′ AU-rich elements of the TNF genomic locus are maintained to assure adequate gene regulation in mice that lack both the TNF and the LT-$\alpha$ genes.[22] Studies on mice expressing a noncleavable TmTNF mutant that replaces the endogenous TNF allele demonstrated an important role for TmTNF in the development of lymphoid structures and in the production of chemokines.[23] LT-$\alpha$, as well as sTNF, is implicated in host defense against mycobacterial infections.[19,24]

In this study, we examine the role of TmTNF in the development of the immune response to *M. bovis* BCG and *M. tuberculosis* infections. We show that TmTNF tg mice were able to efficiently induce a cell-mediated immune response with development of granulomas containing iNOS-expressing cells as also observed in wild-type mice after *M. bovis* BCG infection. In contrast, TNF/LT-$\alpha^{-/-}$ mice showed high sensitivity to BCG infection associated with reduced iNOS activity in infected organs. Although, TmTNF tg mice showed higher survival and enhanced resistance than TNF/LT-$\alpha^{-/-}$ mice to *M. tuberculosis* infection, they died from infection and failed to activate iNOS-producing cells within granulomas. The differential production of iNOS within granulomas may thus represent a key factor in conferring resistance, or susceptibility, to *M. bovis* BCG and *M. tuberculosis* infections.

## Materials and Methods

### Animals

The generation and characterization of TNF/LT-$\alpha^{-/-}$ mice (C57BL/6 × 129/SVEV) transgenic for a noncleavable TmTNF gene have already been described.[22,20] As reported, three mutations (at positions −10, +1, and +10 of the TNF molecule) were introduced into the coding sequence of a genomic clone of mouse TNF. The resulting transgene was under the control of the mouse TNF promotor, and the AU-rich elements at the 3′ end were maintained. TmTNF construct was microinjected into the male pronucleus of TNF/LT-$\alpha^{-/-}$ mice to generate transgenic mice expressing TmTNF in the absence of TNF and LT-$\alpha$.[22] Mice were maintained under conventional conditions in the animal facility of the Medical Faculty, University of Geneva (Geneva, Switzerland), or under P3 conditions at the Pasteur Institute (Paris, France).

### Experimental Infections and Determination of BCG and M. tuberculosis Colony-Forming Units (CFUs)

Mice were infected intravenously with $10^7$ living BCG Pasteur strain 1173 P2 or with $10^5$ CFU of virulent *M. tuberculosis* strain H37Rv. Mortality and weight loss were monitored during the infection. The numbers of viable bacteria were determined by CFU as previously described.[20]

### Histological Analyses

Histological analyses of organs were performed at 4 weeks after BCG and *M. tuberculosis* infections and at necropsy. Liver, lungs, and spleen were fixed in 4% buffered formaldehyde and embedded in paraffin for subsequent hematoxylin and eosin (H&E) and Ziehl-Neelsen staining.

### In Situ Hybridization

An 1108-bp cDNA fragment of the murine TNF gene (positions 1 to 1108; obtained from Genentech Inc., San Francisco, CA) was subcloned into pGEM-2. After linearization of the plasmid, sense and anti-sense RNA probes were prepared using the appropriate RNA polymerase as described previously.[22] *In situ* hybridizations of paraformaldehyde-fixed cryostat sections of lungs were performed with $2 \times 10^5$ cpm of $^{35}$S-labeled RNA probe per $\mu$l of hybridization solution for 18 hours at 48°C.[20]

### Spleen Cell Recovery

Spleen cells from 4-week BCG-infected mice were suspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, treated for 5 minutes with a 0.155 mol/L ammonium chloride/0.010 mol/L potassium bicarbonate solution to lyse the erythrocytes, washed, and resuspended in Dulbecco's modified Eagle's medium plus 10% fetal calf serum as described previously.[20]

### Bronchoalveolar Lavage (BAL)

Mice were anesthetized with Nembutal (0.5 mg/mouse) and the thoracic cavity was opened. The trachea was cannulated and the lungs of each mouse were washed five times with 1 ml of phosphate-buffered saline (PBS) each by gentle instillation. The BAL was spun and the supernatant was removed and stored at −20°C for cytokine analyses.

### Flow Cytometric Analyses

Flow cytometry was performed using one- or two-color staining of spleen cells 4 weeks after BCG infection, and analyzed with a FACSCalibur (BD Biosciences, Mountain View, CA). The antibodies and reagents used were phycoerythrin-conjugated rat anti-CD8 (clone H-35), biotin-labeled rat anti-CD4 (clone H-129), biotin-labeled rat anti-

Mac-1 (clone M1/70), biotin-labeled hamster anti-CD11c (clone HL3), and avidin-phycoerythrin (all from Becton Dickinson-Pharmingen, Heidelberg, Germany). Stainings were performed in the presence of a saturating concentration of 2.4 G2 anti-Fc$\gamma$ RII/III monoclonal antibody.

### *Evaluation of Chemokines and Cytokines in Serum, Bronchoalveolar Fluid, and Lung Protein Extracts*

Blood samples were obtained from retro-orbital sinuses at different time points after infection and bronchoalveolar fluids were obtained by BAL at 4 weeks of BCG infection as described above. Lungs from BCG-infected mice were homogenized in 0.04% Tween 80/saline buffer (125 mg of tissue/ml of buffer). Systemic RANTES regulated on activation normal T cell expressed and secreted monocyte chemoattractant protein (MCP)-1, IFN-$\gamma$, interleukin (IL)-12p70, and IL-12p40 amounts were evaluated by enzyme-linked immunosorbent assay with a sensitivity of 2 to 1000 pg/ml (R&D System, Abingdon, UK, and Immunotec, Belgium).

### *Inducible NOS Activity in Lung and Spleen Extracts*

Evaluation of iNOS activity was done on crude frozen lung and spleen extracts of infected and uninfected mice. Spleens were homogenized in 25 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA, and 1 mmol/L EGTA (125 mg of tissue/ml of buffer). Crude supernatant was obtained by centrifugation of the homogenate at 10,000 $\times$ *g* for 5 minutes. iNOS activity was measured by the ability of supernatant to convert radioactive L-($^{14}$C)-arginine (Amersham Life Science) to L-($^{14}$C)-citrulline as previously described.[20]

### *TNF and iNOS Immunohistochemistry*

Lung sections from paraffin-embedded tissue were rehydrated in PBS and processed for 2 $\times$ 5 minutes in citrate buffer (0.01 mol/L, pH 6.0) in a microwave for iNOS staining and in a pressure cooker for TNF staining. Nonspecific binding sites were blocked in PBS and 1% bovine serum albumin for 30 minutes. For iNOS staining, the sections were incubated with rabbit anti-mouse iNOS (Calbiochem, San Diego, CA) for 60 minutes followed by incubation with biotinylated donkey anti-rabbit antibody (Amersham, Freiburg Germany) revealed by streptavidin-alkaline-phosphatase (Boehringer Mannheim, Rotkreuz, Switzerland). For TNF staining, sections were incubated with a rabbit anti-mouse TNF (Bioreba, Reinach BL, CH) followed by an incubation with a goat anti-rabbit antibody (DakoCytomation, Carpinteria, CA) and revealed with diaminobenzidine and counterstained with H&E. For these two stainings, the preparation incubated without the primary antibody served as the control.

### *Morphometric Analysis*

The sections from immunohistochemistry staining were analyzed by the Metamorph 5.0 software (Universal Imaging, West Chester, PA). An automatic threshold corresponding to the histogram average was applied to generate binary images, and a median filter was used. Results were given in counts (integrating the intensity and the surface of the immunostaining) of positive pixels for iNOS. Objects smaller than a perimeter of 20 pixels were not taken into account.

### *Statistical Analyses*

The unpaired Student's *t*-test was used for all analyses with the exception of survival data which were analyzed by Kaplan-Meier using the log rank test. $P$ values $<0.05$ were considered as statistically significant.

## *Results*

### *Lung Pathologies and TNF Expression in BCG-Infected Mice*

We have previously shown that mice deficient in TNF/LT-$\alpha$ were highly sensitive to BCG infection and die 3 to 8 weeks after infection and that TNF/LT-$\alpha^{-/-}$ mice expressing a transgenic TmTNF form (TmTNF tg) survived for more than 24 weeks.[19,20] In the present study we have examined lung lesions of wild-type, TmTNF tg, and TNF/LT-$\alpha^{-/-}$ mice 4 weeks after infection with BCG ($10^7$ living bacilli). Wild-type and TmTNF tg mice exhibited a similar pattern of pulmonary pathology showing differentiated granulomas containing large macrophages and multinucleated giant cells (Figure 1, A and B). In contrast, TNF/LT-$\alpha^{-/-}$ mice displayed very large lesions and lung necrosis (Figure 1C). As previously described, TNF/LT-$\alpha^{-/-}$ mice developed very few granulomas that did not differentiate and contained high numbers of acid-fast bacilli.[19,20] Lung granulomas from wild-type and TmTNF tg mice contained cells expressing TNF mRNA as evaluated by *in situ* hybridization. Lung tissues showed strong signal for TNF mRNA in cells forming granulomas in wild-type and TmTNF tg mice but not in TNF/LT-$\alpha^{-/-}$ mice (Figure 1; D to F). Moreover, the TNF protein was detectable in the lungs of both wild-type and TmTNF tg mice, preferentially in granulomas and in multinucleated giant cells (Figure 1, G and H).

### *Disregulation of Lung and Systemic Chemokines in BCG-Infected TNF/LT-$\alpha^{-/-}$ Mice and Effect of TmTNF*

Mycobacterial infections induce chemokine activation involved in migration of cells to the site of the infection favoring granuloma formation.[25] It has been shown that TNF is required for regulation of chemokine expression essential for cell recruitment to the granuloma.[26] We have previously examined chemokine activation at the level of

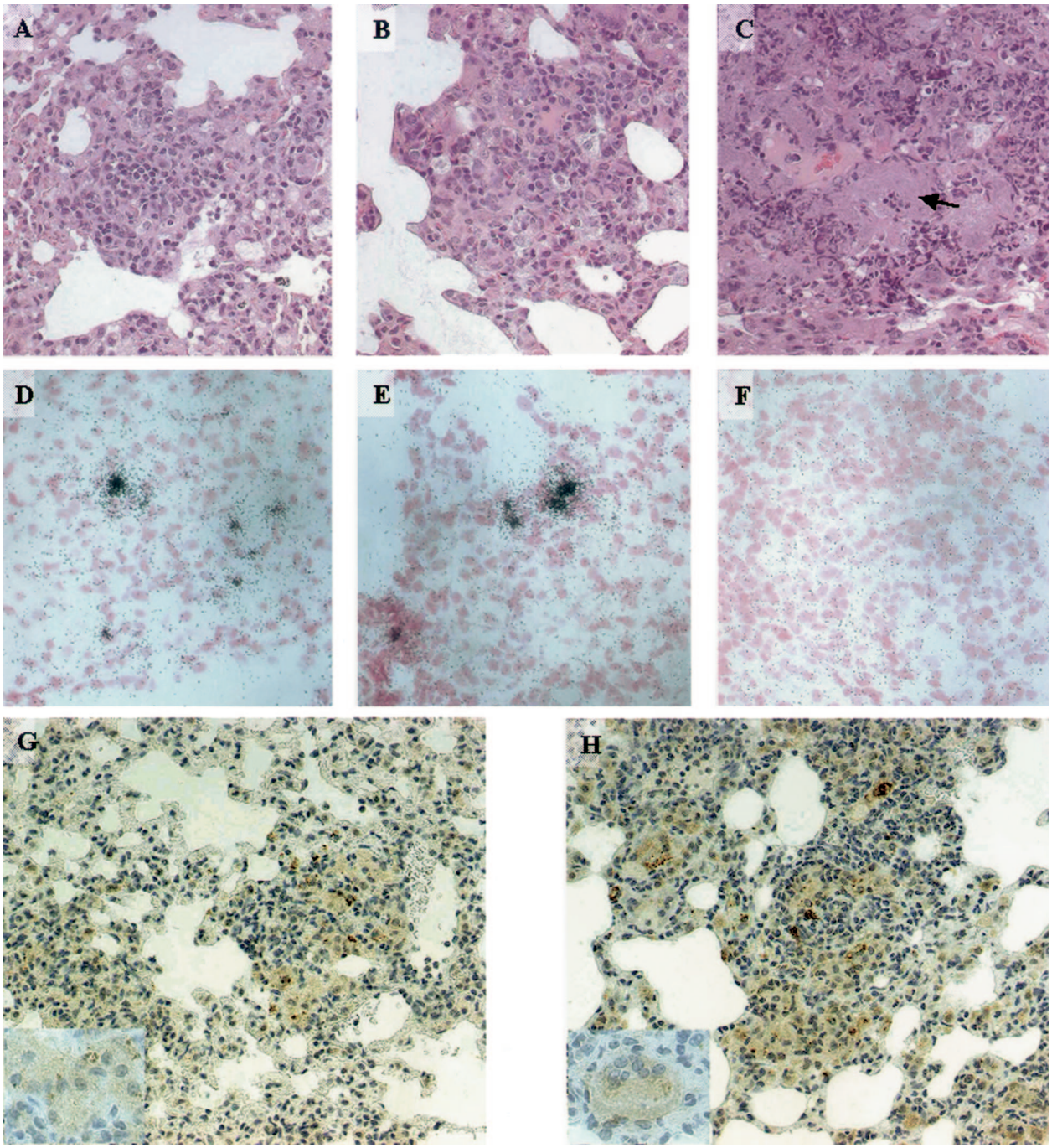


**Figure 1.** Lung granulomas expressing TNF mRNA and protein at 4 weeks of BCG infection. **A–C:** Histological sections from lung (stained with H&E) show well-differentiated granulomas in wild-type (**A**) and TmTNF tg (**B**) mice but disorganized granulomas and necrotic lesions (**arrow**) in TNF/LT-$\alpha^{-/-}$ mice (**C**). **D–F:** *In situ* hybridization with $^{35}$S-labeled TNF RNA probes of lung sections. TNF mRNA-positive cells can be identified by dark silver grains on the lung sections from wild-type (**D**), TmTNF tg (**E**), but not from TNF/LT-$\alpha^{-/-}$ mice (**F**). TNF protein expression was detected by immunohistochemistry in lung granuloma and multinucleated giant cells of wild-type (**G**) and TmTNF tg mice (**H**). These data are representative of two experiments ($n$ = 6 mice per group, **A–C**; $n$ = 3, **D–H**). Original magnifications: ×200 (**A–H**); ×400 (**insets** in **G** and **H**).

RNAs in TNF/LT-$\alpha^{-/-}$ mice and observed that MCP-1, MCP-5, MIP-1$\alpha$, and MIP-1$\beta$ are produced in the lung and liver during BCG infection.[19] We have now evaluated protein levels of MCP-1 and RANTES in the three groups of BCG-infected mice. Indeed, MCP-1 is produced by and acts on monocytes and macrophages, and deficiency in MCP-1 receptor (CCR2) was shown to inhibit granuloma formation after M. *tuberculosis* infection.[27,28] BCG infection induced MCP-1 secretion in serum of wild-type, TmTNF tg, and TNF/LT-$\alpha^{-/-}$ mice indicating that this was independent of TNF release. Wild-type and TNF/LT-$\alpha^{-/-}$ mice had a similar pattern at 2 weeks of infection, however, the presence of TmTNF enhanced MCP-1 secretion (Figure 2A). At 4 weeks of infection, MCP-1 was augmented in both the serum and the BAL fluid of TNF/LT-$\alpha^{-/-}$ mice and this increase was attenuated in TmTNF

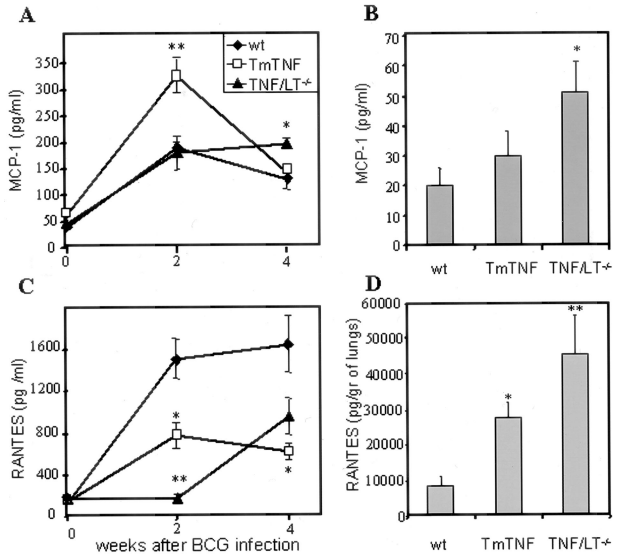


**Figure 2.** Dysregulated systemic and local chemokine release in TNF/LT-$\alpha^{-/-}$ mice and effect of TmTNF on BCG infection. Amounts of MCP-1 (**A** and **B**) and RANTES (**C** and **D**) were measured in serum (**A** and **C**) at different time points, and in BAL (**B**) and in lung proteins (**D**) at 4 weeks of infection in wild-type, TmTNF tg, and TNF/LT-$\alpha^{-/-}$ mice. Data are represented as means pg of protein per ml of fluid ± SEM of 10 mice per group in **A** and **C**, three to five mice in **B**, or in **D** per g of lung, five mice per group. These results are representative of two independent experiments. **Asterisks** indicate statistically significant differences between wild-type and indicated group (*, $P < 0.01$; **, $P < 0.005$).

tg mice, and similar amounts of MCP-1 in BAL fluid of TmTNF tg and wild-type mice were observed (Figure 2B). RANTES, which is produced by a wide variety of cells, including lymphocytes and macrophages,[29,30] and acts on a large group of inflammatory cells such as lymphocytes and macrophages,[31] was measured. RANTES was not released in the serum of TNF/LT-$\alpha^{-/-}$ mice and the amount was lower in the serum of TmTNF tg mice as compared to wild-type mice at 2 weeks after infection, this indicating that RANTES was influenced by TNF. At 4 weeks of infection, systemic RANTES was also decreased in TmTNF tg and TNF/LT-$\alpha^{-/-}$ mice (Figure 2C). In addition, RANTES protein was increased in the lungs of sick TNF/LT-$\alpha^{-/-}$ mice and this was attenuated in the presence of TmTNF (Figure 2D).

## *Alteration of Local and Systemic Th1-Type Cytokines in TNF/LT-$\alpha^{-/-}$ and Effect of TmTNF*

BCG infection induces a Th1-type immune response characterized by the production of IFN-γ and IL-12 required for host defense. We have previously shown that TNF/LT-$\alpha^{-/-}$ mice are unable to activate systemic IFN-γ and IL-12p40 at early infection which is restored by the presence of TmTNF[20]. We have now compared the levels of systemic Th1 cytokines, IFN-γ, IL-12p40, and IL-12p70 (the active form of IL-12), to those in BAL. IFN-γ and IL-12p40 levels were highly increased in both the serum and the BAL fluids of TNF/LT-$\alpha^{-/-}$ mice, this exacerbated secretion of cytokines was attenuated in the presence of the TmTNF form (Figure 3, A to D). TmTNF tg mice showed slight differences and lower levels of IFN-γ when

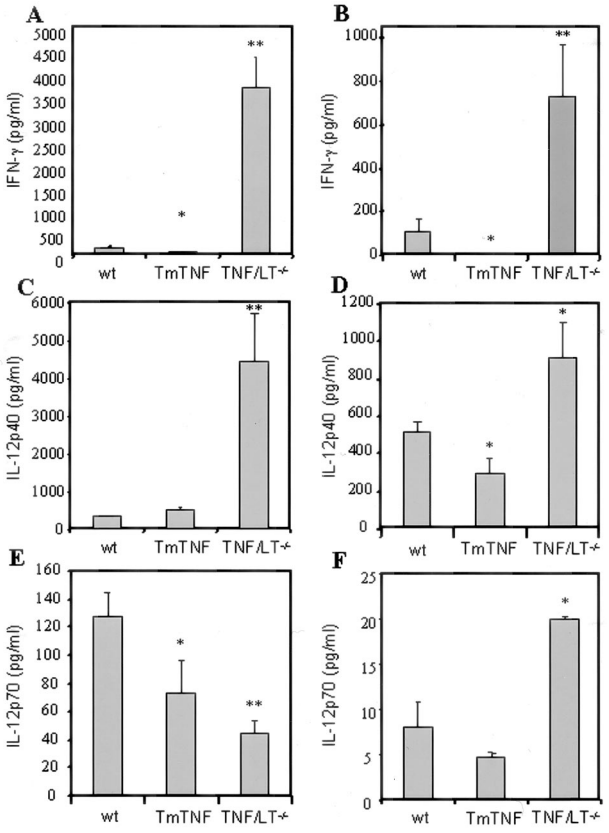


**Figure 3.** Alteration of Th1-type cytokine release in TNF/LT-$\alpha^{-/-}$ mice and effect of TmTNF 4 weeks after BCG infection. Amounts of IFN-γ (**A** and **B**), IL-12p40 (**C** and **D**), and IL-12p70 (**E** and **F**) were measured in serum (**A**, **C**, and **E**) and BAL (**B**, **D**, and **F**) of wild-type (wt), TmTNF tg (TmTNF), and TNF/LT-$\alpha^{-/-}$ (TNF/LT$^{-/-}$) mice. Results are expressed as mean pg of protein per ml of fluid ± SEM ($n = 10$ mice per group for serum cytokines and $n = 4$ mice per group for BAL cytokines). These results are representative of two or three independent experiments. **Asterisks** indicate statistically significant differences between wild-type and indicated group (IFN-γ: *, $P < 0.05$ and **, $P < 0.009$; IL-12p40: *, $P < 0.02$ and **, $P < 0.0001$; IL-12p70: *, $P < 0.03$ and **, $P < 0.0007$).

compared to wild-type mice. The levels of IL-12p70 in serum of TNF/LT-$\alpha^{-/-}$ mice remained lower than those of wild-type mice while in BAL fluid they were higher (Figure 3, E and F). In addition, we have examined the phenotype and frequencies of spleen cell subpopulations 4 weeks after infection (Table 1). The phenotypic analysis of spleen cells revealed that TmTNF tg mice had lower frequencies of Mac-1-positive cells and a slight increase in CD4-positive cells compared to wild-type mice. These changes were markedly enhanced in the TNF/LT-$\alpha^{-/-}$

**Table 1.** Frequencies of Spleen Cell Subpopulations at 4 Weeks of BCG Infection

| Mice | CD11b (Mac-1) | CD11c | CD4 | CD8 |
|---|---|---|---|---|
| Wild type | 24.5 ± 1.6 | 7.1 ± 0.8 | 21.4 ± 0.6 | 6.1 ± 1.3 |
| Tm TNF tg | 19.9 ± 1.1* | 5.2 ± 0.5 | 24.6 ± 2.1 | 5.1 ± 0.8 |
| TNF/LT-$\alpha^{-/-}$ | 14.1 | | 42.6 | 10.6 |

Subpopulations were determined by flow cytometry in the different groups of mice and are expressed as percent means ± SEM of three animals per group except in TNF/LT-$\alpha^{-/-}$ mouse ($n = 1$). *$P < 0.04$.

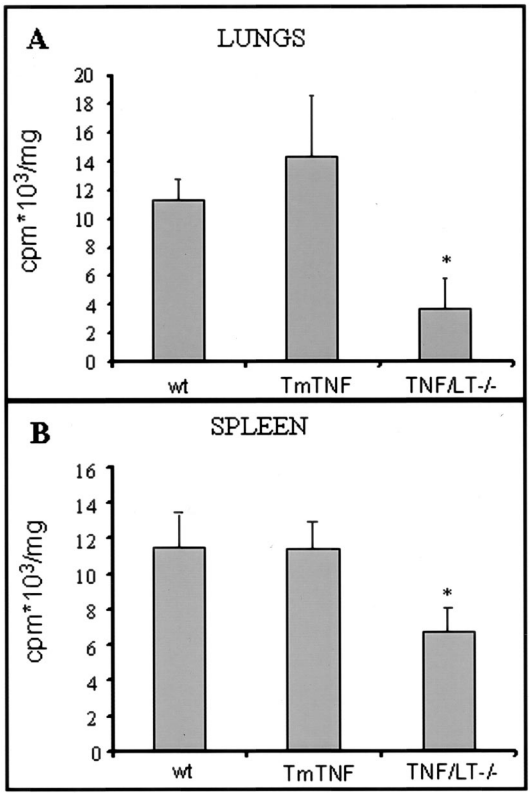


**Figure 4.** iNOS activity is reduced in the lungs and spleen of TNF/LT-$\alpha^{-/-}$ mice but not in those of TmTNF tg mice. iNOS activity was determined in crude lung (**A**) and spleen (**B**) extracts containing the same protein amounts. Data are represented as means ± SEM of cpm/mg of lung proteins ($n = 3$) or spleen proteins ($n = 5$ to 6). **Asterisks** indicate significant differences between wild-type and TNF/LT-$\alpha^{-/-}$ lungs (*, $P < 0.02$) and spleen (*, $P < 0.03$). Experiment has been repeated twice with similar results.

mouse that showed an important reduction in Mac-1 cells and an increase in both CD4 and CD8 T cells (Table 1).

## *Tm TNF Transgenic Mice Efficiently Induce iNOS Activity in Lungs and Spleen but Not TNF/LT-$\alpha^{-/-}$ Mice*

Activation of iNOS in macrophages represents one important bactericidal mechanism to eliminate intracellular bacteria. iNOS is required for protection against BCG infection.[6] The absence of iNOS renders mice susceptible to mycobacterial infections.[6,7] We have evaluated the iNOS activity from crude extracts of lung and spleen at 4 weeks after BCG inoculation by monitoring the conversion of radioactive L-arginine to L-citruline and NO. We observed that iNOS activity was similar in the lung and spleen of TmTNF tg and wild-type mice suggesting that TmTNF is sufficient to activate this enzyme on an avirulent BCG infection (Figure 4). In contrast, TNF/LT-$\alpha^{-/-}$ mice showed a statistically significant reduced activity of iNOS mainly in infected lungs but also in spleen (Figure 4). These data show that iNOS can be activated by both sTNF and TmTNF and that on BCG infection TmTNF is sufficient for bacillus control and elimination.

## *TmTNF Transgenic Mice Show Longer Survival and Enhanced Resistance Than TNF/LT-$\alpha^{-/-}$ but Are More Sensitive Than Wild-Type Mice to* M. tuberculosis *Infection*

Susceptibilities of TNF/LT-$\alpha^{-/-}$ and TmTNF tg mice to *M. tuberculosis* (H37Rv, $10^5$ CFU) infection was assessed by bacterial counts in infected organs at 4 weeks of infection. TmTNF tg mice depicted a significant increase of CFU in the lungs but only a tendency in spleen and liver when compared to wild-type mice (Figure 5A). Bacterial loads of TNF/LT-$\alpha^{-/-}$ mouse infected organs showed a dramatic increase of *M. tuberculosis* living bacilli compared to organs from TmTNF tg mice. Histological analyses of the lung at 4 weeks of *M. tuberculosis* infection revealed numerous granulomas containing large well-differentiated macrophages fused onto multinucleated giant cells in wild-type mice. Furthermore, wild-type mice were able to control recruitment of inflammatory cells to the lungs (Figure 5B) and bacillus growth (Figure 5C). In contrast, TmTNF tg mice exhibited important perivascular and peribronchiolar inflammatory cell accumulation, characterized by the presence of lymphocytes and macrophages occupying the alveolar space leading to lung lesions (Figure 5D). In TmTNF tg mice, the presence of bacilli was limited and contained within granulomas (Figure 5E). Lungs of TNF/LT-$\alpha^{-/-}$ mice had dramatic cellular recruitment, numerous caseous necrotic lesions, and edema accumulation in the alveolar space, which was not observed on BCG infection (Figure 5, F and G). TNF/LT-$\alpha^{-/-}$ mice died between 3 and 7 weeks (median survival time, 31 days) while TmTNF tg mice died between 6 and 13 weeks (median survival time, 56 days) after *M. tuberculosis* infection (Figure 6A). The difference of survival time of TNF/LT-$\alpha^{-/-}$ and TmTNF tg mice was statistically significant ($P < 0.0001$). Comparison of the lung lesions of TmTNF tg and TNF/LT-$\alpha^{-/-}$ mice at necropsy depicted a different pathology. Lung lesions of TmTNF tg mice showed marked inflammatory cell infiltration in the presence of low number of bacilli and absence of caseous necrotic lesions that were the predominant lesions in TNF/LT-$\alpha^{-/-}$ mice associated with very high number of acid fast bacilli (Figure 6; B to E).

## *Spleen and Lung of TmTNF Transgenic and TNF/LT-$\alpha^{-/-}$ Mice Reveal Altered iNOS Expression after* M. tuberculosis *Infection*

Mycobacterial infection induces cytokine expression that triggers bactericidal mechanisms, such as the iNOS activity, which is expressed by macrophages forming bactericidal granulomas. Therefore, we have analyzed the presence of iNOS in spleen and lung at 4 weeks of infection. On *M. tuberculosis* infection, the pattern of iNOS-positive cells in the spleen of TmTNF tg mice showed a reduced number of iNOS-expressing cells compared to wild-type mice and very few cells were stained for iNOS in the spleen of TNF/LT-$\alpha^{-/-}$ mice (Figure 7; A to C). In contrast, the pattern of BCG-infected

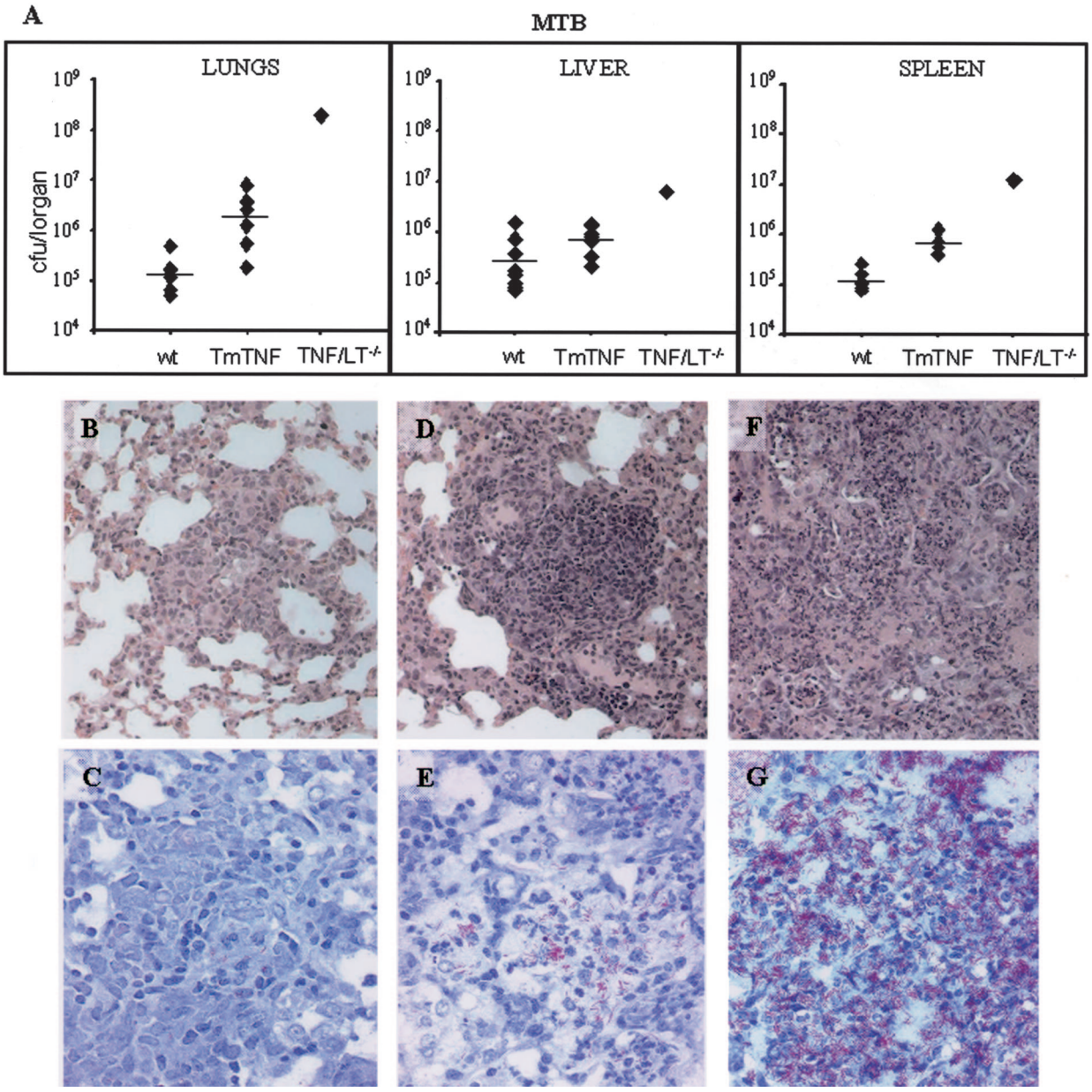


**Figure 5.** TmTNF tg mice are sensitive to *M. tuberculosis* infection and show enhanced cellular recruitment to the lung. **A:** Bacterial loads were determined in lungs, liver, and spleen 4 weeks after intravenous inoculation with $10^5$ CFU of virulent *M. tuberculosis* strain H37Rv. Data represent individual values of infected mice and **horizontal bars** indicate means. A statistically significant increase of CFU in lungs from TmTNF tg mice ($P < 0.01$) when compared to wild-type mice was observed. Each group represents 6 to 10 mice, except TNF/LT-$\alpha^{-/-}$ mouse ($n = 1$). Data from two experiments are shown. **B** to **F:** Enhanced cellular recruitment to the lung of *M. tuberculosis*-infected TmTNF tg mice despite moderate bacillus proliferation. Histological lung sections at 4 weeks after *M. tuberculosis* infection stained with H&E (**B**, **D**, and **F**) and Ziehl-Neelsen (**C**, **E**, and **G**). **B:** Granuloma from wild-type mice with very low number of acid fast bacilli (AFB) (**C**). **D:** TmTNF tg mouse lung granuloma reveals a substantial number of lymphocytes and inflammatory cells, with limited number of AFB(**E**). **F:** TNF/LT-$\alpha^{-/-}$ mouse lung lesion depicts an dramatic cell recruitment and tissue necrosis, associated with an enormous amount of AFB (**G**). These data are representative of two independent experiments, with 8 to 10 mice per group, except TNF/LT-$\alpha^{-/-}$ mice ($n = 1$), which most of them died before 4 weeks of infection. Original magnifications: ×200 (**A**, **C**, **E**); ×400 (**B**, **D**, **F**).

mice was comparable in wild-type and TmTNF tg mice showing cell clusters forming granulomas (Figure 7, D and E), but TNF/LT-$\alpha^{-/-}$ mice exhibited a marked reduction in iNOS-positive cells (Figure 7F). The iNOS-expressing cells were mainly the multinucleated giant cells, as we could observe in the lung of both BCG-infected wild-type and TmTNF tg mice (Figure 7, G and H) and also after *M. tuberculosis* infection. Quantification of iNOS expression in lung and spleen sections from *M. tuberculosi-s* and BCG-infected mice was performed with an algorithm integrating surface and intensity of immunostaining. We found that TmTNF tg mice infected with *M. tuberculosis* displayed a marked decrease in iNOS expression in the lungs (Figure 8A). They also showed a less marked reduction of iNOS-expressing cells in the spleen (Figure 8B). Infection with BCG resulted in comparable iNOS counts in the spleen of both TmTNF tg and wild-type mice but reduction in TNF/LT-$\alpha^{-/-}$ mice. Correlation between

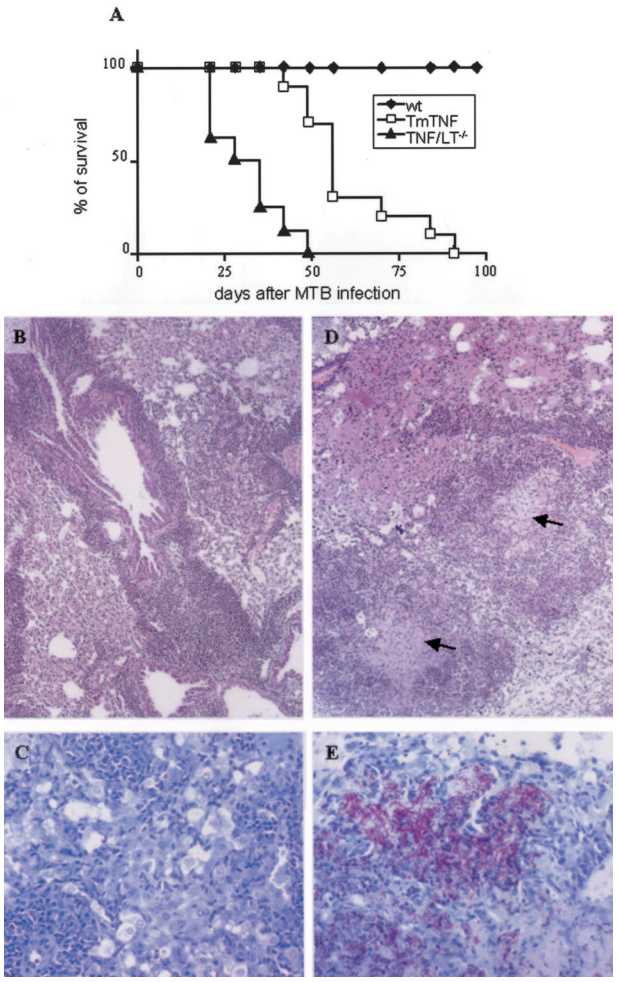


**Figure 6.** Survival curve of *M. tuberculosis*-infected mice and differential lung pathologies of moribund TmTNF tg and TNF/LT-$\alpha^{-/-}$ mice. **A:** Survival curve of wild-type, TmTNF tg, and TNF/LT-$\alpha^{-/-}$ mice after $10^5$ CFU of virulent *M. tuberculosis* strain H37Rv. Each group represents 10 mice, except TNF/LT-$\alpha^{-/-}$ mice ($n = 8$). Data from two experiments are shown. The increase in survival time for TmTNF tg mice was statistically significant when compared to TNF/LT-$\alpha^{-/-}$ mice ($P < 0.0001$). Histological lung sections from moribund TmTNF tg mice (13 weeks after infection) (**B** and **C**) and TNF/LT-$\alpha^{-/-}$ mouse (4 weeks after infection) (**D** and **E**). Sections were stained with H&E (**B** and **D**) or with Ziehl-Neelsen (**C** and **E**). Sick TmTNF tg mouse lung sections reveal a marked inflammatory cell infiltration as main lung pathology (**B**) with a low number of AFB (**C**). In contrast, moribund TNF/LT-$\alpha^{-/-}$ mouse lung pathology was mainly caseous necrotic lesions (**arrows**) (**D**), with a very high number of AFB (**E**). Original magnifications: ×25 (**B**, **D**); ×400 (**C** and **E**).

iNOS staining in tissues and bacillary loads for each individual mouse has been analyzed. We observed that the degree of iNOS staining in the lungs and spleen was directly related to the elimination or the control of bacillary growth (correlation coefficient obtained by scatterplot values from iNOS staining and corresponding CFU were $r = -0.7$ for *M. tuberculosis* and $r = -0.6$ for BCG infection). This suggests a direct association between the capacity to express iNOS in granulomas and the availability to control mycobacterial growth, indicating that the macrophage cell population producing iNOS is critically involved in sensitivity and resistance to mycobacteria.

## *Discussion*

The present work investigates the cell-mediated immune response mounted against BCG and *M. tuberculosis* infections in mice deficient in TNF/LT-$\alpha$, in the presence or absence of a TmTNF form, and provides evidence that regulation of chemokines, Th1-type cytokines, and mainly iNOS is pivotal in mediating sensitivity and resistance to mycobacterial infections. We show that expression of TmTNF can rescue mice from detrimental pulmonary pathology, characterized by enhanced cellular infiltrates, mycobacterial proliferation, and death of the host observed in BCG-infected TNF/LT-$\alpha^{-/-}$ mice. The efficient immune response of TmTNF tg mice against BCG infection was the result of activation and control of chemokines and Th1-type cytokines leading to proper cell recruitment and expansion thus inducing bactericidal mechanisms, eliminating most of bacilli, and preventing their proliferation. Activation of iNOS in cells migrating or differentiating in the lung and spleen of BCG-infected mice correlated with low bacillary loads of TmTNF tg mice whereas the absence of TNF/LT-$\alpha$ resulted in profound iNOS deficiency and inability to clear bacteria leading to a dramatic increase in bacillary burden. We have noticed that in wild-type and TmTNF granulomas, the same cells activating iNOS, including multinucleated cells, also produced TNF and represented the reservoir of bacilli. Our data indicate that susceptibility and resistance to BCG infection depends on two main factors: the availability to induce cells that express TmTNF and iNOS, able to destroy intracellular bacilli before they can multiply and, the capacity to orchestrate the production and the regulation of chemokines and Th1-type cytokines in a TNF-dependent manner.

Homing of leukocytes requires the presence of tissue-associated chemokine gradients and the expression of CCRs on migratory cells.[32] Mycobacterial infection induces chemokine activation and TNF regulates the early chemokine induction necessary for cell recruitment to the infection site.[25,26] Local and systemic amounts of chemokines on BCG infection were measured to address the question of whether absence or presence of TmTNF in TNF/LT-$\alpha^{-/-}$ mice could modify chemokine regulation. We have observed that TmTNF tg mice had increased serum levels of MCP-1 when compared to both wild-type and TNF/LT-$\alpha^{-/-}$ mice. This suggests that TmTNF can influence MCP-1 expression. Absence of MCP-1 or MCP-1 receptor resulted in a transient increase of bacterial load and a reduction in macrophage recruitment without affecting the survival of mice infected with *M. tuberculosis*.[27,33] Interestingly, we observed that increased pulmonary MCP-1 levels correlated with enhanced cell migration and tissue injury in TNF/LT-$\alpha^{-/-}$ mice. TNF/LT-$\alpha^{-/-}$ mice also had a substantial increase of RANTES in the lung, which was attenuated in TmTNF tg mice. We observed that induction of RANTES was dependent on TNF at the early stages of the infection. TmTNF form was previously shown to play a dominant role in chemokine steady-state gene expression, particularly in MCP-1 and RANTES.[23] A recent study has reported that neutralization of TNF with antibodies resulted

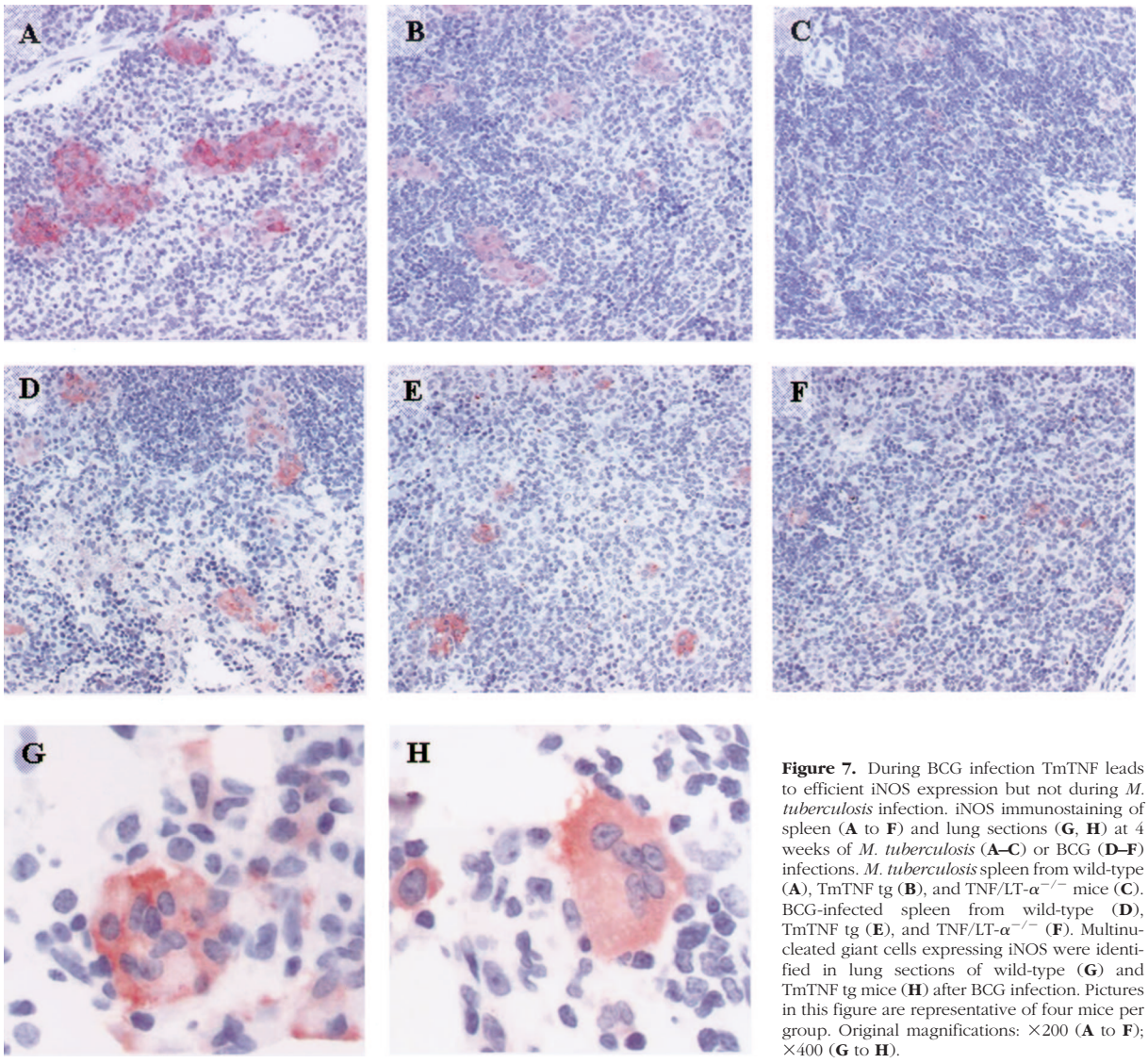


**Figure 7.** During BCG infection TmTNF leads to efficient iNOS expression but not during *M. tuberculosis* infection. iNOS immunostaining of spleen (**A** to **F**) and lung sections (**G**, **H**) at 4 weeks of *M. tuberculosis* (**A–C**) or BCG (**D–F**) infections. *M. tuberculosis* spleen from wild-type (**A**), TmTNF tg (**B**), and TNF/LT-$\alpha^{-/-}$ mice (**C**). BCG-infected spleen from wild-type (**D**), TmTNF tg (**E**), and TNF/LT-$\alpha^{-/-}$ (**F**). Multinucleated giant cells expressing iNOS were identified in lung sections of wild-type (**G**) and TmTNF tg mice (**H**) after BCG infection. Pictures in this figure are representative of four mice per group. Original magnifications: ×200 (**A** to **F**); ×400 (**G** to **H**).

in reduced production of RANTES, but did not affect MCP-1 expression during *M. tuberculosis* infection.[34] Moreover, depletion of RANTES was associated with decreased size of *M. bovis*-induced granuloma.[35] Our results suggest that TmTNF can partially regulate RANTES through infection.

TNF has been considered an essential cytokine contributing to the induction of Th1-type immune response required for cell-mediated resistance against mycobacteria. More recent studies are reconsidering this notion in view of new observations in mice unable to use TNF. One study has previously identified TNF as a negative regulatory cytokine for BCG-induced Th1-type immune responses because it was found to limit an otherwise detrimental Th1-type immune response. Animals unable to produce TNF showed overproduction of Th1-type cytokines in the lungs and peripheral blood, and hyperreactive CD4 and CD8 T-cell responses on BCG infection. Depletion of CD4 and CD8 T cells prevented tissue destruction in TNF$^{-/-}$-infected mice.[21] Previous reports have provided evidence for an anti-inflammatory role of TNF involving its capacity to regulate Th1-type cytokines.[36,37] TNF/LT-$\alpha^{-/-}$ mice did not activate early Th1-type cytokines and then they showed a substantial increase in local and systemic Th1-type cytokines associated with disease severity. This impaired regulation of Th1-type cytokines is likely because of the absence of TNF in TNF/LT-$\alpha^{-/-}$ mice. Indeed, BCG-infected LT-$\alpha^{-/-}$ mice showed regulated IFN-$\gamma$ expression (data not shown), and at 4 weeks of infection, lung lesions were similar to those of wild-type mice.[19] Therefore, LT-$\alpha$ does not appear to influence this enhanced Th1-type immune response.

Although TmTNF tg mice were able to mount an efficient immune response to BCG infection, they were unable to survive to *M. tuberculosis* infection but still showed longer survival than TNF/LT-$\alpha^{-/-}$ mice. Histopathological examinations at 4 weeks of *M. tuberculosis* infection showed a relatively modest increase in bacillary burden compared to wild-type mice. Enhanced cellular infiltration and the resulting tissue lesions, however, were probably

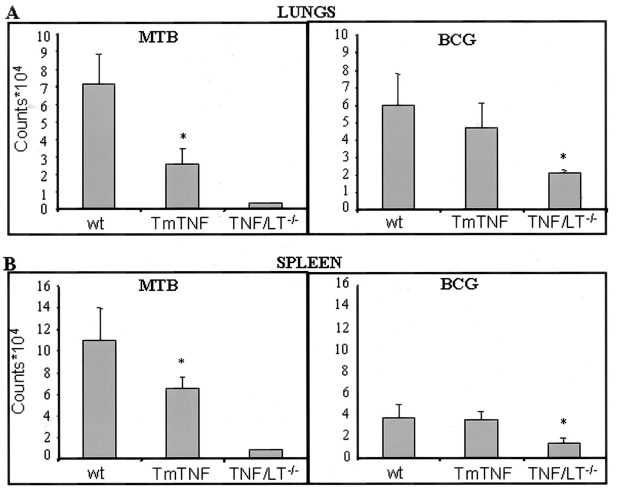


**Figure 8.** TmTNF tg mice are deficient in iNOS-expressing cells in the lung after *M. tuberculosis* but not after BCG infection. Quantification of iNOS immunostaining in the lung (**A**) and spleen (**B**) sections from mice at 4 weeks of *M. tuberculosis* and BCG infection. Data are represented as means of iNOS-positive pixels (±SEM) of three to four mice per group, except TNF/LT-$\alpha^{-/-}$ mouse during *M. tuberculosis* infection ($n = 1$). These results are representative of two independent experiments. **Asterisks** indicate statistically significant differences between the indicated group and wild-type mice (*, $P < 0.01$).

more relevant factors leading to the fatal issue of the infection than the bacillary overgrowth. Moribund TmTNF tg mice did not develop caseous necrotic lesions observed in the lung of TNF/LT-$\alpha^{-/-}$ mice, but massive lymphocytic infiltration was predominant. This indicates that during *M. tuberculosis* infection, TmTNF was not sufficient to attenuate the hyperactivation of lymphocytes leading to exacerbated cell recruitment to the lung, suggesting that the rapid presence of sTNF at the site of infection is necessary to regulate the cell-mediated immune response against virulent mycobacteria. Our results are in agreement with previous reports showing that local inhibition of TNF in the lungs during *M. tuberculosis* infection led to the death of mice because of increased production of proinflammatory factors that induced the tissue destruction in the absence of bacillary overgrowth.[38]

Resistance to BCG and *M. tuberculosis* infections correlated with the capacity to develop cells producing iNOS in the lung and spleen. Indeed, when animals were able to eliminate intracellular bacilli, iNOS was expressed by multinucleated cells within the granulomas and these cells were also immunoreactive for TNF demonstrating that TNF and iNOS were produced by the same cells and that the absence of TNF prevents proper iNOS expression. Moreover, a strong correlation between iNOS expression in activated macrophages and growth containment of both BCG or *M. tuberculosis* in infected organs was found. Activation of iNOS generating NO has been shown to be an important bactericidal mechanism for host defense against mycobacterial infections.[6,7] Activation of iNOS is instrumental in the regulation of the cell-mediated immune response by the multiple activities exerted by NO.[39] NO has been demonstrated to down-modulate T cell responses and the recruitment of inflammatory cells to the sites of infection. We have previously reported that iNOS plays an essential role in down-regulating the immune responses to BCG infection. The absence of iNOS resulted in a dramatic activation of the immune system, up-regulation of TNF, and massive recruitment of lymphoid cells to granulomas, which became caseous necrotic lesions.[6] Bekker and colleagues[40] have shown that TNF may control intracellular bacilli in the absence of iNOS, thus, TNF may act by both iNOS-dependent (killing of bacilli) and iNOS-independent pathways (growth of bacilli). We have observed that during *M. tuberculosis* infection TmTNF tg mice were unable to efficiently activate iNOS-expressing cells in the lung leading to exacerbation of local cell immune response and death from pulmonary lesions because of uncontrolled cell recruitment despite modest bacillary growth. Our findings indicate that both iNOS and TNF may be negative regulators of a detrimental and uncontrolled mycobacteria-induced cell-mediated immune response.

The study of TmTNF activities in mycobacterial infections is important because the treatment of human disease targets TNF with antibodies or with TNF receptors neutralizing sTNF and TmTNF. Recently, several reports demonstrated that the treatment of rheumatoid arthritis and Crohn's disease with TNF inhibitors significantly increases the tuberculosis risk and reactivation of latent tuberculosis.[41–45] Similarly, in murine models, the immune system is unable to control latent tuberculosis without TNF.[46,47] Our results providing evidence that TmTNF is pivotal in resistance against BCG infection and that it partially protects from *M. tuberculosis* infection suggest that the development of selective inhibitors neutralizing soluble but sparing TmTNF would be a promising therapeutic strategy.

## Acknowledgments

We thank Dr. Marie-Laure Santiago-Raber, Dr. Salomé Kantengwa, and Dr. Shozo Izui for critical reading of the manuscript; Ms. G. Levraz, J. Stalder, M. Coassin, P. Gindre, Mr. T. Le Minh, and P. Chavarot for technical analyses; and Y. Donati for important help in statistical analysis.

## References

1. WHO Report: Global Tuberculosis Control. Geneva, World Health Organization, 2003
2. Fine PE: Variation in protection by BCG: implications of and for heterologous immunity. Lancet 1995, 346:1339–1345
3. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, Bloom BR: An essential role for interferon gamma in resistance to Mycobacterium tuberculosis infection. J Exp Med 1993, 178:2249–2254
4. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, Orme IM: Disseminated tuberculosis in interferon gamma gene-disrupted mice. J Exp Med 1993, 178:2243–2247
5. Gordon S, Keshav S, Stein M: BCG-induced granuloma formation in murine tissues. Immunobiology 1994, 191:369–377
6. MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK, Nathan CF: Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc Natl Acad Sci USA 1997, 94:5243–5248

7. Garcia I, Guler R, Vesin D, Olleros ML, Vassalli P, Chvatchko Y, Jacobs M, Ryffel B: Lethal Mycobacterium bovis bacillus Calmette Guerin infection in nitric oxide synthase 2-deficient mice: cell-mediated immunity requires nitric oxide synthase 2. Lab Invest 2000, 80:1385–1397
8. Saito S, Nakano M: Nitric oxide production by peritoneal macrophages of Mycobacterium bovis BCG-infected or non-infected mice: regulatory role of T lymphocytes and cytokines. J Leukoc Biol 1996, 59:908–915
9. Black RA, Rauch CT, Kozlosky CJ, Peschon JJ, Slack JL, Wolfson MF, Castner BJ, Stocking KL, Reddy P, Srinivasan S, Nelson N, Boiani N, Schooley KA, Gerhart M, Davis R, Fitzner JN, Johnson RS, Paxton RJ, March CJ, Cerretti DP: A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells. Nature 1997, 385:729–733
10. Moss ML, Jin SL, Milla ME, Bickett DM, Burkhart W, Carter HL, Chen WJ, Clay WC, Didsbury JR, Hassler D, Hoffman CR, Kost TA, Lambert MH, Leesnitzer MA, McCauley P, McGeehan G, Mitchell J, Moyer M, Pahel G, Rocque W, Overton LK, Schoenen F, Seaton T, Su JL, Warner J, Willard D, Becherer JD: Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha. Nature 1997, 385:733–736
11. Kindler V, Sappino AP, Grau GE, Piguet PF, Vassalli P: The inducing role of tumor necrosis factor in the development of bactericidal granulomas during BCG infection. Cell 1998, 56:731–740
12. Senaldi G, Yin S, Shaklee CL, Piguet PF, Mak TW, Ulich TR: Corynebacterium parvum- and Mycobacterium bovis bacillus Calmette-Guerin-induced granuloma formation is inhibited in TNF receptor I (TNF-RI) knockout mice and by treatment with soluble TNF-RI. J Immunol 1996, 157:5022–5026
13. Garcia I, Miyazaki Y, Marchal G, Lesslauer W, Vassalli P: High sensitivity of transgenic mice expressing soluble TNFR1 fusion protein to mycobacterial infections: synergistic action of TNF and IFN-gamma in the differentiation of protective granulomas. Eur J Immunol 1997, 27:3182–3190
14. Flynn JL, Goldstein MM, Chan J, Triebold KJ, Pfeffer K, Lowenstein CJ, Schreiber R, Mak TW, Bloom BR: Tumor necrosis factor-alpha is required in the protective immune response against Mycobacterium tuberculosis in mice. Immunity 1995, 2:561–572
15. Adams LB, Mason CM, Kolls JK, Scollard D, Kraehenbuhl JL, Nelson S: Exacerbation of acute and chronic murine tuberculosis by administration of tumor necrosis factor receptor-expressing adenovirus. J Infect Dis 1995, 171:400–405
16. Bean AG, Roach DR, Briscoe H, France MP, Korner H, Sedgwick JD, Britton WJ: Structural deficiencies in granuloma formation in TNF gene-targeted mice underlie the heightened susceptibility to aerosol Mycobacterium tuberculosis infection, which is not compensated for by lymphotoxin. J Immunol 1999, 162:3504–3511
17. Roach DR, Bean AG, Demangel C, France MP, Briscoe H, Britton WJ: TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. J Immunol 2002, 168:4620–4627
18. Kaneko H, Yamada H, Mizuno S, Udagawa T, Kazumi Y, Sekikawa K, Sugawara L: Role of tumor necrosis factor-alpha in Mycobacterium-induced granuloma formation in tumor necrosis factor-alpha-deficient mice. Lab Invest 1999, 79:379–386
19. Bopst M, Garcia I, Guler R, Olleros ML, Rulicke T, Muller C, Wyss S, Frei K, Le Hir M, Eugster HP: Differential effects of TNF and LTalpha in the host defense against M. bovis BCG. Eur J Immunol 2001, 31:1935–1943
20. Olleros ML, Guler R, Corazza N, Vesin D, Eugster HP, Marchal G, Chavarot C, Mueller C, Garcia I: Transmembrane TNF induces an efficient cell-mediated immunity and resistance to Mycobacterium bovis bacillus Calmette-Guerin infection in the absence of secreted TNF and lymphotoxin-alpha. J Immunol 2002, 168:3394–3401
21. Zganiacz A, Santosuosso M, Wang J, Yang T, Chen L, Anzulovic M, Alexander S, Gicquel B, Wan Y, Bramson J, Inman M, Xing Z: TNF-alpha is a critical negative regulator of type 1 immune activation during intracellular bacterial infection. J Clin Invest 2004, 113:401–413
22. Mueller C, Corazza N, Trachsel-Loseth S, Eugster HP, Buhler-Jungo M, Brunner T, Imboden MA: Noncleavable transmembrane mouse tumor necrosis factor-alpha (TNF alpha) mediates effects distinct from those of wild-type TNF alpha in vitro and in vivo. J Biol Chem 1999, 274:38112–38118
23. Ruuls SR, Hoek RM, Ngo VN, McNeil T, Lucian LA, Janatpour MJ, Korner H, Scheerens H, Hessel EM, Cyster JG, McEvoy LM, Sedgwick JD: Membrane-bound TNF supports secondary lymphoid organ structure but is subservient to secreted TNF in driving autoimmune inflammation. Immunity 2001, 15:533–543
24. Roach DR, Briscoe H, Saunders B, France MP, Riminton S, Britton WJ: Secreted lymphotoxin-alpha is essential for the control of an intracellular bacterial infection. J Exp Med 2001, 193:239–246
25. Rhoades ER, Cooper AM, Orme IM: Chemokine response in mice infected with Mycobacterium tuberculosis. Infect Immun 1995, 63:3871–3877
26. Roach DR, Bean AG, Demangel C, France MP, Briscoe H, Britton WJ: TNF regulates chemokine induction essential for cell recruitment, granuloma formation, and clearance of mycobacterial infection. J Immunol 2002, 168:4620–4627
27. Peters W, Scott HM, Chambers HF, Flynn JL, Charo IF, Ernst JD: Chemokine receptor 2 serves an early and essential role in resistance to Mycobacterium tuberculosis. Proc Natl Acad Sci USA 2001, 98:7958–7963
28. Scott HM, Flynn JL: Mycobacterium tuberculosis in chemokine receptor 2-deficient mice: influence of dose on disease progression. Infect Immun 2002, 70:5946–5954
29. Schall TJ, Jongstra J, Dyer BJ, Jorgensen J, Clayberger C, Davis MM, Krensky AM: A human T cell-specific molecule is a member of a new gene family. J Immunol 1988, 141:1018–1025
30. Devergne O, Marfaing-Koka A, Schall TJ, Leger-Ravet MB, Sadick M, Peuchmaur M, Crevon MC, Kim KJ, Schall TT, Kim T, Galanaud P, Emilie D: Production of the RANTES chemokine in delayed-type hypersensitivity reactions: involvement of macrophages and endothelial cells. J Exp Med 1994, 179:1689–1694
31. Schall TJ, Bacon K, Toy KJ, Goeddel DV: Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. Nature 1990, 347:669–671
32. Cyster JG: Chemokines and cell migration in secondary lymphoid organs. Science 1999, 286:2098–2102
33. Kipnis A, Basaraba RJ, Orme IM, Cooper AM: Role of chemokine ligand 2 in the protective response to early murine pulmonary tuberculosis. Immunology 2003, 109:547–551
34. Algood HM, Lin PL, Yankura D, Jones A, Chan J, Flynn JL: TNF influences chemokine expression of macrophages in vitro and that of CD11b+ cells in vivo during Mycobacterium tuberculosis infection. J Immunol 2004, 172:6846–6857
35. Chensue SW, Warmington KS, Allenspach EJ, Lu B, Gerard C, Kunkel SL, Lukacs NW: Differential expression and cross-regulatory function of RANTES during mycobacterial (type 1) and schistosomal (type 2) antigen-elicited granulomatous inflammation. J Immunol 1999, 163:165–173
36. Hodge-Dufour J, Marino MW, Horton MR, Jungbluth A, Burdick MD, Strieter RM, Noble PW, Hunter CA, Pure E: Inhibition of interferon gamma induced interleukin 12 production: a potential mechanism for the anti-inflammatory activities of tumor necrosis factor. Proc Natl Acad Sci USA 1998, 95:13806–13811
37. Marino MW, Dunn A, Grail D, Inglese M, Noguchi Y, Richards E, Jungbluth A, Wada H, Moore M, Williamson B, Basu S, Old LJ: Characterization of tumor necrosis factor-deficient mice. Proc Natl Acad Sci USA 1997, 94:8093–8098
38. Smith S, Liggitt D, Jeromsky E, Tan X, Skerrett SJ, Wilson CB: Local role for tumor necrosis factor alpha in the pulmonary inflammatory response to Mycobacterium tuberculosis infection. Infect Immun 2002, 70:2082–2089
39. Bogdan C: Nitric oxide and the immune response. Nat Immunol 2001, 2:907–916
40. Bekker LG, Freeman S, Murray PJ, Ryffel B, Kaplan G: TNF-alpha controls intracellular mycobacterial growth by both inducible nitric oxide synthase-dependent and inducible nitric oxide synthase-independent pathways. J Immunol 2001, 166:6728–6734
41. Gardam MA, Keystone EC, Menzies R, Manners S, Skamene E, Long R, Vinh DC: Anti-tumour necrosis factor agents and tuberculosis risk: mechanisms of action and clinical management. Lancet Infect Dis 2003, 3:148–155

42. Gomez-Reino JJ, Carmona L, Valverde VR, Mola EM, Montero MD: Treatment of rheumatoid arthritis with tumor necrosis factor inhibitors may predispose to significant increase in tuberculosis risk: a multicenter active-surveillance report. Arthritis Rheum 2003, 48:2122–2127
43. Wagner TE, Huseby ES, Huseby JS: Exacerbation of Mycobacterium tuberculosis enteritis masquerading as Crohn's disease after treatment with a tumor necrosis factor-alpha inhibitor. Am J Med 2002, 112:67–69
44. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, Siegel JN, Braun MM: Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. N Engl J Med 2001, 345:1098–1104
45. Long R, Gardam M: Tumour necrosis factor-alpha inhibitors and the reactivation of latent tuberculosis infection. CMAJ 2003, 168:1153–1156
46. Mohan VP, Scanga CA, Yu K, Scott HM, Tanaka KE, Tsang E, Tsai MM, Flynn JL, Chan J: Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: possible role for limiting pathology. Infect Immun 2001, 69:1847–1855
47. Botha T, Ryffel B: Reactivation of latent tuberculosis infection in TNF-deficient mice. J Immunol 2003, 171:3110–3118